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Review

Use of live bacterial vaccine vectors for antigen delivery: potential and limitations

Eva Medina, Carlos Alberto Guzmán *

Department of Microbial Pathogenesis and Vaccine Research, Division of Microbiology, GBF-German Research Center for Biotechnology, Mascheroder Weg 1, D-38124 Braunschweig, Germany

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Abstract

Most infectious agents are restricted to the mucosal membranes or their transit through the mucosa constitutes a critical step in the infection process. Therefore, the elicitation of an efficient immune response, not only at systemic, but also at mucosal level, after vaccination is highly desirable, representing a significant advantage in order to prevent infection. This goal can be only achieved, when the vaccine formulation is administered by the mucosal route. However, soluble antigens given by this route are usually poorly immunogenic. Among the available approaches to stimulate efficient mucosal responses, the use of bacterial carriers to deliver vaccine antigens, probably, constitutes one of the most successful strategies. The potential and limitations of the most extensively studied bacterial carrier systems will be discussed.

Keywords: Carriers; Mucosal immunity; Vaccines

1. Introduction

Vaccination constitutes the most cost-effective tool for the prophylaxis of infectious diseases. Most pathogenic microorganisms are either restricted to the mucosal membranes or need to transit them during the early steps of the infection [1]. Therefore, the elicitation of an efficient immune response at mucosal level after immunization is highly desirable [2]. This may result in a more efficient protection against infection, also facilitating the eradication of diseases for which humans are the only reservoirs. Due to the apparent compartmentalization of the systemic and mucosal immune system, parenterally administered vaccines are less effective in protecting against mucosal pathogens [3,4]. Thus, administration of immunogens through the mucosal route is required to achieve full protection [3,4]. In addition, the use of the mucosal route is associated by itself with a considerable number of additional advantages (Table 1).

Different strategies can be used to deliver vaccine antigens by the mucosal route. Among them, the use of bacterial carriers, probably, constitutes the most studied strategy (Table 2). Delivery of vaccine antigens by live bacterial carriers has resulted in the elicitation of effective humoral and cellular responses, at the level of both systemic and mucosal compartments [5].

2. Vaccine delivery systems based on live bacterial vectors

Live vaccine vectors are delivered at the mucosal surface, place in which the onset of infection takes place and the first defense line is laid. On the basis of a common mucosal system, the generated immune response will not only be present at the specific inductive site in which antigen priming took place, but also at remote mucosal sites [3,4]. In addition to the general advantages mentioned in Table 1, the use of bacterial carriers is associated with other benefits, such as low

* Corresponding author. Tel.: +49-531-6181558; fax: +49-531-6181411.

E-mail address: cag@gbf.de (C.A. Guzmán).

batch preparation costs, facilitated technology transfer following development of the prototype, increased shelf-life and stability in the field respect to other formulations (e.g. subunit vaccines), easy administration and low delivery costs. Taken together, these advantages make this strategy particularly suitable for mass immunization programs. By using a carrier as source for a recombinant antigen, the presence of any additional products from the pathogen, which might be reactogenic, is ruled out (e.g. potential traces of co-purified products in acellular vaccines). Thus, the carrier operationally becomes an equivalent of a subunit recombinant vaccine. This may in turn facilitate the critical evaluation of antigen-related side effects during clinical phases, when well-characterized carriers are used.

Both attenuated and commensal microorganisms have been successfully used as carriers for vaccine antigens (Table 3). The use of attenuated pathogens seems particularly attractive, since protection against the pathogen itself and immune responses specific for the heterologous antigen(s) can be simultaneously achieved. The efficacy of an attenuated vaccine carrier relies on a subtle balance between minimal reactogenicity and maximal immunogenicity. The background of the carrier strain used in the formulation, the type of mutation selected to achieve attenuation, and the intrinsic properties of the immunogen seem to be crucial in determining the extent and quality of the immune response elicited [6,7] (Table 4). The bacterial species, which have been considered suitable as antigen delivery systems and exhibit a satisfactory immunogenicity profile will be briefly reviewed.

Table 1
Advantages associated with vaccine administration by the mucosal route

Mucosal administration	Parenteral administration
Low reactogenicity	Generally higher reactogenicity than mucosal
High acceptance/increased compliance	Pain = reduced acceptance/reduced compliance
Inductive sites are targeted	Inductive sites are not targeted
Systemic and mucosal responscs are stimulated	Only systemic responses are stimulated
Protection against disease and infection	Mainly protection against disease
No cross contamination/increased safety	Potential risk of cross contamination
Easy administration/simple logistics	Requirement of trained personnel
Low delivery associated costs	Higher delivery costs

Table 2
Strategies used to administer vaccines by the mucosal route

Mucosal antigen delivery systems
• live viral or bacterial vaccine carriers
• mucosal adjuvants
• viral particles
• ISCOMs
• liposomes
• microparticles
• transgenic plants

2.1. *Listeria monocytogenes*

L. monocytogenes is a Gram-positive intracellular pathogen, which has been considered as the prototype for the elicitation of MHC class I-restricted immune responses. This microorganism can stimulate strong cell-mediated responses against its own proteins or co-expressed antigens. The ability of *L. monocytogenes* to breach into the cytoplasm of infected cells allows that the expressed proteins gain access to the endogenous antigen-processing pathway, facilitating epitope presentation in the context of MHC class I molecules [8,9]. This type of immune response is considered to be particularly important for the clearance of intracellular bacterial pathogens, viruses, tumors and parasites [8,10–13].

Different mutations in virulence-associated determinants of *L. monocytogenes* have been exploited to develop attenuated strains amenable for use as live carriers [9]. Attenuated *L. monocytogenes* vaccine carriers have been used to stimulate strong cellular immune responses (mainly CD8 + CTL) against different viruses [9,14–18] and for the immunotherapy of cancer [19–22]. Vaccination with bacteria expressing tumor-associated antigens has been shown to protect animals, not only against lethal challenge with tumor cells, but also to cause regression of preestablished macroscopic tumors in an antigen-specific T cell-dependent manner [19–22].

Table 3
Live bacterial vectors which have been successfully used as antigen delivery systems

Attenuated mucosal pathogens	Commensal strains
L. monocytogenes	*S. gordonii*
Salmonella spp.	*Lactobacillus* spp.
V. cholerae	*Staphylococcus* spp.
Shigella spp.	
M. bovis BCG	
Y. enterocolitica	
B. anthracis	

Table 4
General factors to be considered to optimize the immune responses stimulated by bacterial carriers

Carrier-related factors	Antigen-related factors
Selection of the carrier	Intrinsic properties of the antigen
Specific background strain	Expression system
Attenuating mutation	Antigen-display form
Level of attenuation	Stabilization of the recombinant phenotype
Stabilization of the attenuated phenotype	Co-expression of modulating molecules
Establishment of the optimal dosage	Vaccination schedule

2.2. Salmonella spp.

Salmonella are intracellular pathogens that remain restricted to the endosomal compartment of eukaryotic cells, resisting non specific killing mechanisms [23]. The introduction of defined non-reverting mutations affecting critical virulence factors from *Salmonella* has been used to generate an array of live vaccine carriers. Although many attenuated mutants were constructed and characterized for virulence in the mouse model, only a few of them have been evaluated as vaccine carriers. Mutants deficient in the biosynthesis of aromatic amino acids or purines, production of adenylate cyclase or cAMP receptor protein, carrying mutations affecting the global regulatory system *phoP/phoQ* or lacking the DNA adenine methylase, have been the most widely characterized [24–30]. These mutants are excellent carriers for vaccine antigens from other bacteria [31,32], viruses [33,34], parasites [35,36], and tumors [37], being able to stimulate strong systemic and local immune responses against the corresponding antigens. Therefore, vaccine prototypes based on attenuated *Salmonella* strains can be employed, when effective humoral responses are required (e.g. to achieve clearance of extracellular pathogens) and also for clinical situations in which Th1 helper and/or cytotoxic effector cells are needed (e.g. viral diseases and tumors).

2.3. Yersinia enterocolitica

Y. enterocolitica is an enteric pathogen able to invade intestinal tissues, resisting host clearance mechanisms. The invasion of intestinal cells by *Y. enterocolitica* is largely dependent on the presence of a virulence plasmid [38], which codes for the synthesis of several virulence determinants. These proteins are synthesized during the invasion phase and are able to stimulate a strong antibody response. Recombinant *Y. enterocolitica* strains have been used as carriers for heterologous antigens, leading to the elicitation of efficient and protective immune responses at systemic and mucosal levels [39–41]. The antigen-specific secretory IgA triggered by *Y. enterocolitica* was found, not only in the intestine, but also in the respiratory tract [42].

2.4. Commensal microorganisms

Lactobacillus belong to the normal flora from the gut and the genitourinary tract, and have been widely used as probiotics in the food industry. In addition to their wide acceptance by the public and their intrinsic safety profile, a large variety of biological and immunomodulatory properties make them attractive candidates as carriers for mucosal vaccination [43]. Different *Lactobacillus*-based vaccine prototypes have been developed and administered by the mucosal route, leading to the elicitation of both mucosal and systemic immune responses against the expressed antigens [44,45]. *Streptococcus gordonii* is also a commensal microorganism, which has been exploited as carrier. Different recombinant antigens from human pathogens have been successfully expressed and delivered using this system [46–49].

Many viral and bacterial pathogens have their port of entry at the level of the genital mucosa. Therefore, the elicitation of an efficient local response at the target site constitutes an important goal for the development of efficient vaccines against sexually transmitted diseases. Both *S. gordonii* and *Lactobacillus* spp. can be employed to develop vaccines against sexually transmitted pathogens. Recombinant strains of *S. gordonii* expressing the V3 domain of the gp120 protein of the HIV-1 virus or the E7 protein of human papillomavirus have been engineered. Antigen-specific local (secretory IgA) and systemic (humoral and cellular) responses were observed in vaccinated monkeys [48,49].

2.5. Other bacterial carrier systems

Mycobacterium bovis BCG represents the most widely used attenuated bacterial vaccine all over the world. Therefore, its potential use as bacterial carrier for heterologous antigens appears as extremely attractive. The stimulation of protective immune responses by prototypes expressing HIV antigens can be mentioned among the studies showing more promising results [50,51].

The *Bacillus anthracis* toxinogenic Sterne strain is currently used as a live veterinary vaccine against anthrax. Attenuated strains of *B. anthracis* expressing heterologous antigens seem to exhibit a significant potential as carriers for veterinary vaccines [52–54].

As previously mentioned, attenuated vaccine strains developed to protect against intestinal pathogens can also be used as carriers. The expression of protective antigens from other microorganisms would, thereby,

facilitate the development of multicomponent vaccines [55]. As an example, a live candidate against shigellosis was developed using the attenuated cholera vaccine strain *Vibrio cholerae* 103 Hg^R as a carrier for the *Shigella sonnei* O-antigen [56]. Vaccination with this prototype resulted in the elicitation of high titres of *S. sonnei* O-antigen-specific antibodies in vaccinated animals [57]. Other examples have been provided by candidates based on the attenuated *Shigella flexneri aroD* strain SFL124, which was used to express antigens from *Shigella dysenteriae* I. The administration of the generated prototypes by mucosal route led to the stimulation of protective responses [58–60].

3. Use of live bacterial vectors as vehicles for the delivery of DNA vaccines

DNA vaccination is a new approach for the elicitation of efficient responses by employing genes encoding the vaccine antigen, rather than using the proteins themselves [61]. The fact that many attenuated bacterial vectors are specifically targeted to the antigen presenting cells at inductive sites suggested that they could also be used to specifically deliver DNA to them. In this context, the recombinant antigen will not be expressed by the carrier itself, but rather the gene encoding the vaccine antigen will be delivered to host cells. Thus, to the general advantages of the mucosal delivery, the benefits associated with DNA vaccination should be added (e.g. technical easiness, post-translational modifications of the synthesized product, etc.). Microorganisms which have the capacity to access the cytoplasm of infected cells, such as *Shigella* and *Listeria*, have been used to deliver DNA constructs in which the expression of the recombinant antigen was under the control of a eukaryotic promoter [62,63]. The in vitro efficiency of the *Listeria*-based system has been increased by using conditional self-destructing carriers engineered to produce a phage lysin [63]. Attenuated *Salmonella* strains can also be used for the delivery of DNA to eukaryotic cells [64–66]. It was demonstrated that *Salmonella*-based vaccines can be used to prevent both infections and tumors by specifically targeting antigen presenting cells, and that this approach can also be used to correct genetic defects in macrophages [64–66].

4. Modulation of the immune responses stimulated by live bacterial carriers

The successful resolution of infections caused by microbial pathogens is determined, to a great extent, by the balance achieved between the different cellular populations which are stimulated. CD4+ Th cells can differentiate into two distinct types of effector cells, Th1 cells, which regulate cell-mediated immunity, and Th2 cells, which mainly regulate humoral immune responses [67]. It has been demonstrated that the elicitation of Th2-type responses is particularly important to achieve protection against certain parasitic infections [68]. In contrast, Th1-type responses enhance the microbicidal activity of macrophages, facilitating the clearance of intracellular pathogens [8,69]. Therefore, evaluation and selection of the most adequate strategy to stimulate the required quality of immune response is an essential step during the vaccine design phase. The rational exploitation of attenuated bacterial strains as antigen delivery system may require the development of strategies to stimulate appropriate effector populations according to the specific needs [70].

The type of the microorganism which will be selected as a carrier is a critical factor to be considered. In fact, the intracellular compartment where the carrier resides has a decisive impact on the type of T cell sub-population to be stimulated following vaccination. CD8+ T cells are the main cellular population stimulated during infection with *L. monocytogenes* [8]. On the other hand, CD4+ T cells seem to be the main effector population triggered during *Salmonella* infection [71], although a role for CD8+ T cells has also been demonstrated [72,73].

The quality of the evoked immune response not only depends on the general properties of the carrier, but also on the nature of the specific mutation affecting the virulence properties. This feature has been exploited to fine-tune the quality of the immune response elicited by the carrier strain [6,7]. Therefore, the availability of a well-characterized panel of mutants able to promote specific types of immune responses (e.g. different Th patterns) might allow to modulate the obtained responses by selecting the most appropriate mutant according to the specific application. Interestingly, the quality of the immune response generated by the vaccine antigen is not entirely determined by the selected carrier or the nature of the immunogen, but it is also influenced by the particular promoter chosen to drive antigen expression [74]. The Th pattern response stimulated by the carrier was shifted from a mixed Th1/Th2 to a dominant Th1 by just using different promoters to control the expression of the vaccine antigen, leading to an improved efficacy of the prototype [74].

The form of antigen display by the carrier and the compartment where the recombinant antigen is expressed can also influence the quality of the triggered response. Depending on the topology, the antigen can gain access to either the MHC class I or II degradation pathway, which in turn will influence the generation of antigen-specific CD4+ and/or CD8+ T cells. Using appropriated expression systems, the vaccine antigen can be either secreted into the environment, displayed attached to the carrier surface or retained into the

cytoplasm [15,20,36,58,59,75–79]. The secretion apparatus of the hemolysin of *Escherichia coli* has been the most extensively used, leading to an overall increment of the efficacy of the vaccine candidates [59,75–79]. However, secretion has been shown to be dependent on the folding rate of the specific protein [78]. This problem can be solved by the expression of only selected epitopes, which play a critical role in the elicitation of a protective response.

As mentioned above, the global quality of the immune response stimulated against the heterologous antigen is, to a large extent, predetermined by the dominant type of immunity promoted by the carrier itself. However, this can be modified by co-administering or co-expressing specific modulating cytokines, which can modify the polarization pattern of the stimulated T cells [80].

5. Problems associated with the use of live bacterial carriers

5.1. Reversion to virulence and/or reactogenicity

The carrier strains used as backbone for the generation of vaccine prototypes should exhibit an adequate safety profile. Strains belonging to the same bacterial species and carrying identical attenuating mutations may show different in vivo performances as a result of their strain-specific virulence profile. In addition, when attenuated pathogens are used as carriers, the stability of the attenuated phenotype should be ensured. This can be achieved by selecting specific target genes/loci for which singular inactivation results in attenuation. Subsequently, independent deletions should be introduced in two or more of these genes. This would eliminate or make negligible the risk of reversion to virulence, as a result of recombination events or horizontal gene transfer.

For vaccine prototypes based on attenuated enteropathogenic bacteria, the therapeutic window may be significantly different in endemic versus non endemic areas. Due to pre-existing immunity, the vaccine dose required to trigger efficient responses in non-endemic areas may be inefficacious in countries in which wild type strains are normally circulating. Conversely, the established dosage for endemic areas might be reactogenic in non-endemic areas. A potential solution for this dilemma might be the presentation of the vaccine in different formulations, according to the geographic area.

5.2. Stability of the recombinant phenotype

The use of bacterial strains as vaccine carriers for heterologous antigens raises the issue of selecting the most appropriate expression strategy to optimize the production of the recombinant antigen, thereby, warranting carrier competitiveness, reproducibility and efficacy. The use of plasmids is often associated with instability of the recombinant phenotype. Furthermore, the presence of antibiotic resistance markers in strains which will be delivered under uncontained conditions is not desirable. The use of low copy number vectors and/or their modification by inclusion of killing systems, partition functions and non antibiotic selection markers have improved their performance and safety profiles [59,60,79,81]. Stability can also be achieved by integrating the cassettes coding for the heterologous antigens into the chromosome of the carrier strain [59,60]. However, the low expression levels obtained using a monocopy gene dosage, may result to be insufficient to stimulate efficient responses [82]. To circumvent this problem, alternative expression strategies, such as the use of in vivo activated promoters, have been proposed [74,83].

5.3. Horizontal gene transfer

The expression of particular vaccine antigens by non pathogenic commensals or environmental microorganisms, may increase their virulence for human or animal populations. In this context, the release of a live vector containing such gene(s) may represent an environmental risk. Therefore, it might be necessary for specific applications, to establish systems to minimize the possibility of horizontal gene transfer from the vaccine strain to members of the mucosal flora or environmental microorganisms. This can be achieved by using different strategies, such as the incorporation of conditional lethal systems [84,85].

5.4. Pre-existing immunity

The possibility that prior exposure to the bacterial vector might compromise the efficacy of the initial or additional vaccine constructs, which are based in the same microorganism, has been demonstrated by using *Salmonella* spp. [86]. The immune responses elicited against the heterologous antigen were dramatically impaired in situations of pre-existing immunity against the carrier strain. By using different carrier species or bacterial serotypes for the preparation of different formulations, this problem can be circumvented. Alternatively, the establishment of an optimal window for the re-administration of the carrier may contribute to eliminate this bottleneck.

5.5. Host genetic factors

Host genetic factors may modulate the immune response stimulated by the live bacterial carrier itself or

the expressed vaccine antigens. This aspect should also be taken into consideration, when live vaccine candidates are developed and/or tested. In this regard, it has been shown that the diversity of the mouse H-2 haplotype can determine the development of an efficient CD4+ Th1 response against a recombinant antigen expressed by *S. typhimurium* [87]. In mice, early bacterial replication following infection with *S. typhimurium* is controlled by the gene *Nramp1* (natural-resistance-associated macrophage protein), which might also influence responses to recombinant antigens expressed by this bacterium. Experiments using mice congenic for *Nramp1* evidenced that in those carrying the resistance allele a dominant Th1 response was stimulated, whereas congenic mice carrying the susceptibility allele of *Nramp1* developed a Th2 predominant response [88].

6. Conclusions

The use of live bacterial carriers constitutes a powerful tool to achieve an efficient delivery of either vaccine antigens or DNA vaccine constructs. Almost unlimited possibilities are offered for the exploitation of this system in the context of clinical applications, such as immunotherapy and immunoprophylaxis of infectious diseases, cancer or chronic processes. To the multiple advantages associated with the use of the mucosal route, the amplification effect derived from persistent antigen production, increased stability and low production costs should be added. In the last few years, many defined attenuated mutants and expression systems have been generated and tested. These studies performed both in animals and humans gave us a significant knowledge in terms of the immune responses stimulated by bacterial carrier-based vaccines and their overall efficacy to prevent disease. This constitutes a solid platform for the development of new prototypes based in this technology in the years to come.

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